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Molecular Detection of Aspergillus Fumigatus in A Sample of Iraqi Patients

Abbas M. Ammari[1*] and Fakhar AL-Nisaa Z. Kareem[2]
[1]Forensic Sciences Department, College of Science, Diyala University, 23001, Iraq
[2]Biology Department, College of Science, Diyala University, 23001, Iraq

Author Designation: [1]Associate Professor, [2]Researcher

*Corresponding author: Abbas M. Ammari (e-mail: abbas@uodiyala.edu.iq).

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Abstract: **Background:** *Aspergillus fumigatus* is the most common causes of Aspergillosis. *CYP51* and *calmodulin* are considered virulence genes related with the progression of invasive Aspergillosis. **Objectives:** To detect the role of mutations for *CYP51* and *Calmodulin* genes in azoles resistance of *A. fumigatus* isolated from different clinical samples. **Methods:** One hundred fifty samples were collected (asthma, cutaneous and sinusitis) patients. *Aspergillus fumigatus* was identified using C-Zapek Dox agar medium incubating at 37°C for 72 hours. The susceptibility of *A. fumigatus* isolates was tested toward Amphotericin B (20 µg) and itraconazole (10 µg). Ten isolates of *A. fumigatus* were tested to detect of substitution mutations for both genes. **Results:** The percentage of *A. fumigatus* isolated from diabetic Aspergillosis patients and non diabetic aspergillosis patients were 20.5% and 14.5% respectively. The molecular weight of *CYP51* and *Calmodulin* genes for *A. fumigatus* isolates were 140 bp and 461 bp, respectively. All *A. fumigatus* isolates for sinusitis and asthma patients have substitution mutations in both genes. For non diabetic patients, one isolate of sinusitis sample has substitution mutation for *CYP51* gene only. **Conclusions:** *CYP51* gene play a role in azole resistance of *A. fumigatus* among Aspergillosis diabetic patients.

Key Words: Aspergillus Fumigatus, Aspergillosis, *CYP51* Gene, *Calmodulin* Gene

INTRODUCTION

Aspergillus is a filamentous fungus, it is disseminated in different environments like air, soil, water and food, and cause many problems when it spores inhaled by the host especially in those whom immunocompromised persons. the lung is the most organ effect by Aspergillus spores [1]. Aspergillus fumigates is the most pathogenic among other species of Aspergillus, it characterized with wide distribution through the release of spores and it optimal grows between 25°C - 30°C, *A. fumigates* associated with high mortality rate (90%) among aspergillosis infections. In spite of its high mortality, invasive aspergillosis still understudied and underdiagnosed when it compared with other disorders [2,3].

Allergic bronchopulmonary aspergillosis (ABPA) is a lung fungal infection, it caused because the hypersensitivity reaction of *Aspergillus* antigens that colonized into the airways of humans. This disease is commonly among patients with bronchial asthma and those having cystic fibrosis [4]**.** Aspergillosis is not common in skin caused by Aspergillus species; it occurs by contagious or by hyphal transmission from blood [5].

Aspergillus sinusitis accounts around 9% of all rhinosinusitis cases. Polyps and thick secretions, patients with Aspergillus sinusitis are characterized with neutropenia, diabetes mellitus, excessive use of antibiotics, and immunosuppressive drugs, all these factors may be predisposing for Aspergillus sinusitis [6]. Diabetic patients have more than 25% a higher risk of developing to infect with pulmonary aspergillosis, *A. fumigates* is the most causative agent of this infection due to it could invade the pulmonary vasculature and cause thrombosis, pulmonary infarctions among diabetic patients [7]. In immunocompetent humans, they can remove *A. fumigatus* spores and prevent Aspergillosis problems. However, in immunocompromised patients especially whom use immunosuppressive drugs, *A. fumigatus* colonize the target organ and may develop Aspergillosis ([8].

Azole derivatives represent the first choice for aspergillosis treatment. itraconazole is widely used among other azoles in treat of invasive aspergillosis [9]. Azoles inhibit of the Cyp51 proteins which act with lanosterol-14 alpha demethylase in synthesis of *A. fumigatus'* ergosterol. The presence of mutations could affect the inhibition azoles and lead to decrease of these antifungals [10].

METHODS

Samples Collection

One hundred fifty samples (swabs and scraps) were collected from patients with (asthma, cutaneous and sinusitis) problems who attended respiratory, dermatology and ENT consulting clinics at Baquba Teaching Hospital for a period extended from 2nd of January 2024 to the 30th of June 2024. The samples were collected from diabetic and non diabetic patients (88 and 62 samples), respectively. The clinical samples were diagnosed by consultant physicians.

Conventional Detection of Aspergillus Fumigatus

The conventional detection of *Aspergillus fumigatus* was done by culturing the isolates on Sabouraud Dextrose Agar (SDA) medium incubating at 37C for 1-2 weeks. Next, the isolates were stained using Lactophenol cotton blue onto glassed slide. For additional identification, a single hypha of isolates was sub-cultured on and C-Zapek Dox agar (CZA) medium with incubating at 37°C for 48-72 hrs. [11].

Antibiotics Susceptibility Test

The susceptibility of *A. fumigatus* isolates was done toward commonly antibiotics used in treatment of fungal infections using disk diffusion method. *Aspergillus fumigatus* isolates cultured on Mueller Hinton agar medium were tested toward Amphotericin B (20 µg) and itraconazole (10 µg) as described by researchers [12].

Detection of Virulence Genes of *Aspergillus Fumigates* Isolates Using PCR Method

Extraction of Aspergillus Fumigates DNA: Genomic DNA of *Aspergillus fumigatus* isolates was extracted according to the protocol of BIO-pure Extraction kit for fungal DNA extraction.

Polymerase Chain Reactions Method

The PCR constitute of 25 µLs as total volumes which were made from Go Taq Green Master Mix (12.5 µLs) containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM each of dATP, dTTP dGTP, and dCTP, 3 mM MgCl2, bluish loading dye to analyze PCR product using agarose gel electrophoresis), forward primers (1 µLs), reverse primers (1 µLs), DNA (2 µLs), and nuclease free water (8.5 µLs). Sequences of primers each of *CYB51* and *Calmodulin* genes for *A. fumigates* isolates and the program was done using PCR thermo cycler with condition (30 cycles) are mentioned in Table 1 and 2.

Agarose gel electrophoreses

After PCR. carrying out, agarose gel electrophoreses was done to detect the existence and integrity for the PCR. product. A gram of the powder (agarose) was resolved in fifty mls of buffered Tres Borate EDTA. (T.B.E.) to be agarose gel at pH 8. Next, mixture was dissolved using microwave. one microliter of ethidium bromide (10 mg/ ml) was added to agarose solution, with stirred to mix and mixture was cooled at 45°C. After comb fixed in one cm away from the margin, the agarose solution was put in tray of gel. After solidifying of gel, the comb was sided and the gel tray was put in the tank which filled with 0.5X buffered TBE. Five microliters of *A. fumigatus* DNA were disorder with 2 µl of bromophenol blue dye (loading buffer). Samples were put onto the gel wells, the electrical power was turn on 100 volt/mAmp for 1 hr. DNA mobile from (-) cathode pole to (+) anode. Stained bands visible by UV transiluminator at 350 nm.

Molecular Detection of Virulence Genes for Aspergillus fumigatus Isolates using Gene Sequencing Method

For gene sequencing, twelve isolates of *A. fumigatus* in both directions were sent to Microgen Inc., South Korea to detect of substitution mutations for both genes. The sent isolates were two isolates for each studied groups among diabetic and non-diabetic patients. The sequencing data of targeted gene that received from Microgen Inc. were assembled and translated to contig format and text document using Contig Express module of Vector NTI 9.0 program. All the reference nucleotide sequences of targeted genes of identified microbial vaginitis from (www.ncbi) and aligned using Clustal W method of MEGA4 program.

Table 1: The primer of Virulence Genes for Aspergillus Fumigatus Isolates

Name primers	Primers sequence	Products size (bp.)	Ref.
CYP51-F	5`-CTTTTTCGACTGCCGCGC-3`	140	[13]
CYP51-R	5`-AGGCGTAGTGAGTGGAGA-3`		
Calmodulin-F Calmodulin-R	5'-CCGAGTACAAGGAGGCCTTC -3'	461	[14]
	5'–CCGATAGAGGTCATAACGTGG-3'		

Table 2: PCR Program Virulence Genes for Aspergillus Fumigatus Isolates

Steps	Temp. (°C)	Time	No. of Cycle
Initial denaturation	94°C	5 mins.	1
Denaturation	94°C	45 secs.	30
Annealing	58°C (52°C for Calmodulin gene)	45 secs.	
Extension	72°C	2 min	
Final extension	72°C	5 mins.	1

Ethical Approval

The protocol, the study information form was confirmed by local ethic board based on the decision 1674 in 4\1\2024.

RESULTS

Among the diabetic patients, the percentages of *A. fumigatus* isolated from asthma, cutaneous and sinusitis aspergillosis were 19.2% (5 out of 26), 13.8% (4 out of 29) and 27.3% (9 out of 33) respectively. Whereas, among the nondiabetic patients, the percentages of *A. fumigatus* isolated from asthma, cutaneous and sinusitis aspergillosis were 8.3% (2 out of 24), 19.1% (4 out of 21) and 17.6% (3 out of 17), respectively as shown in Table 3. No statistically significant difference was revealed in aspergillosis among diabetic and non-diabetic patients (p>0.05). The macroscopic examination of *A. fumigates* isolates grow as a velvety, suede like surface with greenish blue colored mycelia. the microscopic appearance of *A. fumigatus* showed smoothed wall conidiophore with swollen vesicle with phialids coating on the upper half of its surface.

Antibiotics Susceptibility Test for Aspergillus Fumigatus Isolates

Disc diffusion method (Kirby-Bauer) was used to perform susceptibility test of two antibiotics (Amphotericin B and itraconazole) against all *A. fumigates* (18 isolates for diabetic patients and 9 isolates for non-diabetic patients). The results were compared with CLSI stander (CLSI, 2017). Among diabetic patients of aspergillosis, five isolates of *A. fumigatus* (27.8%) were resistant toward both antibiotics. Whereas, among non-diabetic patients of aspergillosis, three isolates of *A. fumigatus* (33.3%) were resistant toward both antibiotics. No statistically significant difference was revealed in resistance of *A. fumigatus* isolates toward both studied antifungals (p>0.05) (Table 4).

Molecular Identification of Aspergillus Fumigatus Isolates

Detection the Virulence Genes of *A.* Fumigatus Isolates Using Singleplex PCR: The molecular weight of *CYP51* and *Calmodulin* genes for *A. fumigatus* isolates were 140 bp and 461 bp, respectively. This was indicated sign for successes reaction. Figure 1 A and B.

Detection of Substitution Mutations of Virulence Gene for *A. Fumigatus* Isolates by Gene Sequencing

After performing of the alignment between the amino acid reference sequences of *CYP51 and Calmodulin genes for A. fumigatus* isolates and amino acids sequences of ten isolates

Table 3: Percentage of Aspergillus Fumigatus Isolates Concerning Study Groups

	Diabetic patients		Non diabetic patients	
Study groups	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Asthma	5 (27.8%)	21 (30.0%)	2 (7.4%)	22 (41.5%)
Cutaneous	4 (22.2%)	25 (35.7%)	4 (14.8%)	17 (32.1%)
Sinusitis	9 (50.0%)	24 (34.3%)	3 (11.1%)	14 (26.4%)
Total	18 (20.5%)	70 (79.5%)	9 (14.5%)	53 (85.5%)
P value	0.115			

Table 4: Antibiotics susceptibility Test for Aspergillus Fumigatus Isolates

		Amphotericin B		Itraconazole		
Study groups		Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)	Total (%)
Diabetic aspergillosis patients	Asthma	2 (20%)	3 (17.6%)	4 (26.7%)	1 (8.3%)	5 (18.5%)
	Cutaneous	1 (10%)	3 (17.6%)	2 (13.3%)	2 (16.6%)	4 (14.8%)
	Sinusitis	3(30%)	6 (35.3%)	5 (33.3%)	4 (33.2%)	9 (33.3%)
Non diabetic aspergillosis patients	Asthma	1(10%)	1 (5.9)	2 (13.3%)	0 (0.0%)	2 (7.4%)
	Cutaneous	2(20%)	2 (11.8%)	1 (6.7%)	3 (24.9%)	4 (14.8%)
	Sinusitis	1(10%)	2 (11.8%)	1 (6.7%)	2(16.6%)	3 (11.1%)
Total		10 (100%)	17 (100%)	15 (100%)	12(100%)	27 (100%)
P value		0.959		0.768		

Table 5: Percentage of Aspergillus Fumigatus Isolates Concerning Their Ages

			Age groups				
Study groups			<20 years	20-29 years	30-39 years	40-49 years	>50 years
Diabetic patients	Asthma	Co.	0	2	2	0	1
		%	0.0%	66.7%	50.0%	0.0%	20.0%
	Cutaneous	Co.	0	0	1	2	1
		%	0.0%	0.0%	25.0%	50.0%	20.0%
	Sinusitis	Co.	2	1	1	2	3
		%	100%	33.3%	25.0%	50.0%	60.0%
	Total	Co.	2	3	4	4	5
		%	100%	100%	100%	100%	100%
	P value		0.423				
Non diabetic patients	Asthma	Co.	1	0	1	0	0
		%	100%	0.0%	25.0%	0.0%	0.0%
	Cutaneous	Co.	0	0	1	2	1
		%	0.0%	0.0%	25.0%	100%	100%
	Sinusitis	Co.	0	1	2	0	0
		%	0.0%	100%	50.0%	0.0%	0.0%
	Total	Co.	1	1	4	2	1
		%	100%	100%	100%	100%	100%
	P value		0. 476				

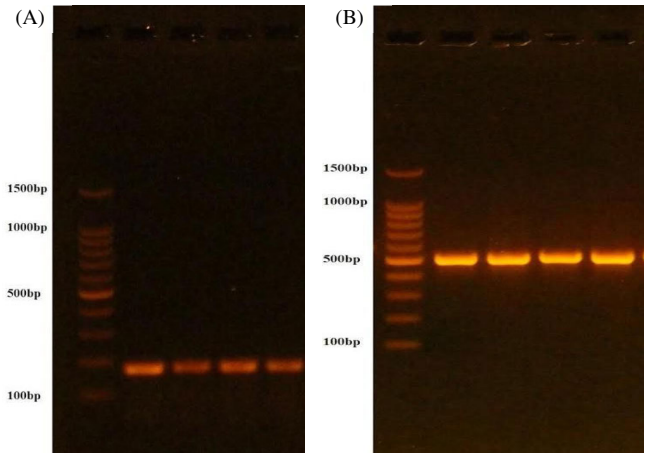

Figure 1: Agarose Gel Electrophoreses of PCR Products of: (A) *Cyp51* And (B) *Calmodulin* Genes for *A. Fumigatus* Using 1% Agarose Gel At 100 Volt/Mamp For 1 Hr. Lane M 100 Bp Dna Ladder, (1-4) Pcr Products

of this study. For diabetic patients, all isolates of *A. fumigatus* for sinusitis and asthma samples have substitution mutations for both genes. For non diabetic patients, one isolate of sinusitis samples has substitution mutation for *CYP51* gene only. All isolates of cutaneous samples for both genes did not have any substitution mutations among the studied groups.

Relation of Study Groups, Percentage of Isolates with Age Groups

Among diabetic patients, the age group (40-49) years old was the most infected with *A. fumigatus* that isolated from cutaneous and sinusitis infections with a percentage (50.0%). Whereas, among non diabetic patients, the age groups (40-49) and (30-39) years old were the most infected with *A. fumigatus* that isolated from cutaneous and sinusitis infections with the percentages (100.05) and (50.0%), respectively. No statistically significant difference was revealed between aspergillosis and age groups in both studied groups ($p>0.05$) (Table 5).

DISCUSSION

Invasive aspergillosis especially that caused *A. fumigatus* affect immunocompromised patients include those with hematologic malignancies, patients who underwent lung or liver cirrhosis, diabetic patients, using of antibiotics for prolong periods and those with organ transplant [15,16]. The truly and soon diagnosis of aspergillosis and rapidly determination of suitable antifungal drug led to improve survival significant [17]. Molecular detection methods so important in identification of pathogenic fungi in special methods that detect about the substitution mutation of some virulence genes like gene sequencing method. For example, *CYP51* gene in Aspergillosis caused by *A. fumigatus* play an important role in mechanism of triazole actions due to the mutations that encoded triazoles targeted enzymes which necessary for ergosterol biosynthesis in fungal plasma membranes [18]. This result disagreed with [19,20] whom isolated *A. fumigatus* with sputum more than sinusitis and cutaneous samples with a percentage (68.75%). This genus is widespread airborne mold pathogens, which led to the increasing cases of Aspergillosis during past decades [21]. Patient populations may be played a role in the incidence of invasive Aspergillosis and explanation the varies in these results. Also, Calmodulin gene was used for identifying *A. fumigatus* from other species in cases of Aspergillosis [22]. Azoles are widely used in treatment of human and animal infections which caused by *A. fumigatus*, in protection of crops against diseases caused by fungi or in wood preservation as biocides. Therefore, azoles present as residues from these materials in many closet areas such as surfaced water, groundwater and sediments), these may be an opportunity for development of resistant Aspergillus isolates toward antifungals [23]. Studies indicated that many azoles act an inhibit *CYP51* and *calmodulin* genes which mean that these antifungals can interacted with iron atoms of the heme group of *CYP51* and the closely amino acids become in reverse and competitive manners, that way challenge the usual substrates for binding site [24]. These results were closed with [25] which revealed that *CYP51* gene mutations were associate with *A. fumigatus* resistant to azoles, and made of possible roles in maintain some levels of resistance when *CYP51* gene functions are weak. Also, these results were closed with results of [26] which find that the percentage of azoles resistance was 3.2% among isolates of *A. fumigatus* and out of these resistant isolates, 78% were *A. fumigatus* had mutation of *Cyp51* gene. Whereas, these results disagreed with [22] which revealed that no mutation with *calmodulin* gene for *A. fumigatus* clinical isolates. Aspergillosis was invasive in immunocompromised patients that used corticosteroid for prolong periods or patients with neutropenia [27]. *Aspergillus fumigatus* has ability to biosynthesize different from secondary metabolites like fumagillins,

fumitoxin, gliotoxin, these metabolites may cause serious health hazard and involved in impairing the host immune system [28].

CONCLUSIONS

Depending on the findings, our results concluded that diabetic patients were more infected with Aspergillosis than non diabetic patients and the genes of *CYP51 and Calmodulin* play an important role in the resistance of *A. fumigatus* towards studied antifungals.

Recommendations

This study is recommended to detect the relationship between virulence genes of *A. fumigatus* isolated from Aspergillosis patients. Also, we recommended to detect the role of *CYP51 and Calmodulin* genes of Non-fumigatus Aspergillus species in in the pathogenicity of invasive Aspergillosis or Aspergilloma.

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